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# STUDIES ON THE MECHANISMS OF DELAYED AND STIMULATED DELAYED FLUORESCENCE OF CHLOROPLASTS

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SUMMARY

1. Simultaneous measurements were made of prompt and delayed fluorescence of spinach chloroplasts upon acidification followed by a sudden jump of pH towards a higher value and upon a sudden increase in the ionic strength of the medium. It was found that the stimulation of delayed fluorescence caused by the above treatments was not paralleled by a significant increase of prompt fluorescence. This indicates that this stimulation is due to an increase in the rate of the back reaction of primary photoproducts of Photoreaction 2 which is not caused by an increase in concentration of reduced electron acceptor brought about by "reserved" electron flow.

2. Experiments with mixtures of salts supported the hypothesis that the stimulation of delayed fluorescence upon addition of salt is due to the establishment of a diffusion potential which is positive on the inside with respect to the outside of the thylakoid membrane and which is caused by different permeabilities of cations and anions.

3. The stimulation of light emission due to a pH jump towards a higher value after acidification was enhanced by the presence of KCl (low concentration) and valinomycin. This indicates that a negative membrane potential is developed by the efflux of protons, which tends to diminish the stimulation of light emission and which is decreased by exchange diffusion of $K^+$.

4. A decrease of the pH after illumination from pH 7.8 to values between 7 and 3 to 4.5, depending on the experimental conditions, gave a decrease in the yield of delayed and prompt fluorescence. Increase in permeability to hydrogen ions caused by damage to the thylakoid membrane or by addition of uncouplers of phosphorylation resulted in a stimulation of luminescence.

5. The stimulated delayed fluorescence and the rapid, but not the slow phase of "normal" delayed fluorescence (measured 2 sec and 5 msec after preillumination, respectively) were dependent upon the integrity of the thylakoid membrane, as indicated by a high sensitivity towards treatment with the detergent Triton X-100.

6. The above and other observations are tentatively explained by a model in which the reduced primary electron acceptor and oxidized donor of System 2 are

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)-ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tricine, *N*-tris (hydroxymethyl)methylglycine.

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located on the outside and inside of the thylakoid membrane and react in a pH-dependent equilibrium:

$$Q^- + H^+ \rightleftharpoons QH, \text{ and } ZH^+ \rightleftharpoons Z + H^+$$

Light emission occurs upon back reaction of $Q^-$ and $ZH^+$. This reaction is stimulated by a membrane potential and by a difference of proton concentration which are positive on the inside with respect to the outside of the thylakoid.

## INTRODUCTION

The phenomenon of delayed light emission by chlorophyll after preillumination of photosynthetic material has been the object of several investigations (*e.g.* refs. 1–4) after its discovery by STREHLER AND ARNOLD[5] in 1951.

LAVOREL[6,7] and CLAYTON[8] have studied the relation between prompt and delayed fluorescence. LAVOREL obtained evidence that the intensity of delayed fluorescence ("luminescence") of algae is proportional to the yield of prompt fluorescence and also under certain conditions, proportional to the difference between the actual yield and the yield of fluorescence when all reaction centers of Photosystem 2 are in the trapping state, the so-called "variable fluorescence". The latter effect suggested a proportionality with the concentration of the reduced primary electron acceptor $Q^-$, which was in line with the hypothesis [1,5,9] that delayed fluorescence is caused by a recombination of the primary photoproducts of System 2. CLAYTON concluded that the luminescence yield of chloroplasts is proportional to what he called "live fluorescence". Since he assumed that almost all the fluorescence measured when the reaction centers are in the trapping state is due to "dead fluorescence", and the yield of "live fluorescence" was assumed to be only slightly larger than the yield of variable fluorescence, there is some similarity between the observations and conclusions of LAVOREL and of CLAYTON. According to both concepts, the yield of delayed fluorescence is approximately proportional to the yield of variable fluorescence.

It was recently found that the intensity of delayed fluorescence can be stimulated considerably by various treatments after the preillumination. With isolated chloroplasts such a stimulation can be brought about by a so-called acid–base transition[10] (acidification followed by a jump towards higher pH) or by a sudden increase in the ionic strength of the suspension[11,12]. In a previous paper[12] it was shown that the salt-induced stimulation of luminescence is specifically dependent upon the cations and anions used and upon the presence of agents such as gramicidin and valinomycin that increase the membrane permeability of certain monovalent cations. On the basis of these experiments the luminescence stimulation was assumed to be due to the generation of a potential difference across the thylakoid membrane, caused by a difference in cation and anion permeabilities.

This paper is the report of a further study of the above-mentioned phenomena by means of a modified apparatus which enabled simultaneous registration of luminescence and fluorescence. Evidence will be given that supports the above-mentioned hypothesis. Experiments on luminescence stimulation induced by acid–base transition indicate that this stimulation is due to the establishment of a difference in electrochemical potential between the inside and the outside of the thylakoid, which is com-

posed of a pH gradient that stimulates, and a membrane potential that tends to depress light emission.

The results will be discussed in terms of a scheme in which both the primary electron donor and acceptor exist in two forms that are in a pH-dependent equilibrium with each other and are located at different sides of the thylakoid membrane.

## MATERIALS AND METHODS

### *Isolation of chloroplasts*

The chloroplasts were obtained from market spinach or from spinach grown in a greenhouse and picked shortly before the experiments. For some experiments the leaves were washed and macerated in a blendor at 0–4° in a solution of pH 7.8 containing 0.4 M sucrose and either 0.05 M Tris–HCl, 0.05 M *N*-tris(hydroxymethyl)-methylglycine (Tricine)–KOH or 0.05 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)–KOH. The homogenate was filtered through two layers of nylon or perlon gauze (mesh width 60–80 $\mu$m), and the filtrate was centrifuged at 1000 $\times$ $g$ for 5 min. The chloroplast pellet was resuspended in the same buffered sucrose solution and stored at 0° in the dark. Chloroplasts isolated in this way will be referred to as "normal chloroplasts".

For other experiments chloroplasts were isolated from freshly picked leaves by a method described by KRAAYENHOF[13]. Briefly, the leaves were cut into small pieces in cold 0.4 M sucrose with 0.05 M Tricine, brought to pH 7.8 with NaOH or KOH, and the chloroplasts were gently squeezed from the leaf fragments by means of a ribbed perspex mortar and a ribbed teflon roller, filtered through two layers of perlon net (56 $\mu$m) and centrifuged at high speed (up to 8000 $\times g$) for a short time. After gentle resuspension in the same buffer as used for the isolation the chloroplasts were stored at a high concentration ($1 \cdot 10^{-3}$–$5 \cdot 10^{-3}$M chlorophyll) on ice in the dark. This procedure yielded at least 50 % Class I chloroplasts (as defined by SPENCER AND UNT[14]). Chloroplasts prepared by this method will be referred below to as "Class I chloroplasts".

The chlorophyll concentrations were determined according to WHATLEY AND ARNON[15].

### *Procedure*

The procedure for measuring luminescence and fluorescence was as follows. After proper dilution a sample of the chloroplast suspension was illuminated in a preillumination cuvette, subsequently mixed with an equal volume of acid or buffer solution, depending on the type of experiment, and transferred to a syringe. The contents of this syringe, and of an identical one containing appropriate mixing solution, were rapidly emptied, mixed and transferred to a measuring cuvette. Luminescence and fluorescence were simultaneously recorded. Unless otherwise indicated, this mixing occurred 3 sec after preillumination. For control experiments the second syringe contained the same buffer solution as that in which the chloroplasts were suspended.

### *Apparatus*

The preillumination cuvette was a perspex vessel of 1 mm thickness, and contained 1.5 ml of the chloroplast suspension. It was illuminated for 5 sec by red light of

saturating intensity, $2 \cdot 10^{-9}$ Einstein $\cdot cm^{-2} \cdot sec^{-1}$, transmitted by a Balzers Calflex C and B40 645 nm interference filter and a Schott RG2, 3-mm glass filter. A second identical vessel contained an equal volume of buffer or acid solution. After simultaneous opening of two taps in the exit tubes, the contents of the vessels were mixed (first mixing) and transferred by compressed air within one sec into a stainless steel syringe (Fig. 1).

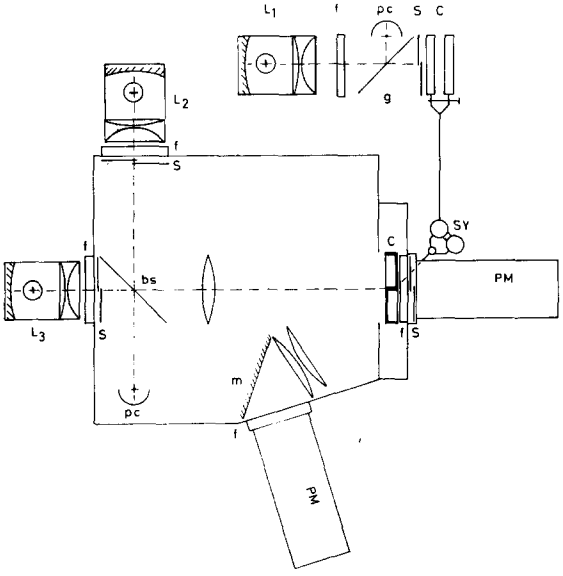


Fig. 1. Apparatus (partly schematic) for measuring delayed and prompt fluorescence. PM, photomultipliers; m, mirrors; c, cuvettes; f, filters; s, shutters; g, a glass plate; bs, a beam splitter. The lamps $L_1$ and $L_2$ served to provide the preillumination and the fluorescence excitation light respectively. $L_3$ provided preillumination in the experiments of Table III. The light intensities were measured by means of photocells (pc). The preilluminated chloroplast suspension was transferred to a syringe (sy), subsequently mixed with the contents of a second one and transferred to the measuring cuvette. Further details are given in the text.

An identical syringe, containing the same volume of buffer, base, salt or acid solution, was mounted in a parallel position close to the first one. The exits of these syringes were connected *via* stainless steel tubes, and a Y-shaped tap to a common delivery pipe attached to the measuring cuvette (second mixing). Opening of the tap automatically caused the expulsion of the contents of the syringes by means of an air-driven piston attached to both plungers. The speed of the flow in the exit-tubes was such that turbulent mixing occurred in the delivery tube.

The measuring cuvette ($4 \times 4 \times 0.2$ cm$^3$) was divided into two optically isolated halves with perspex windows facing two different photomultipliers (EMI-type 9558). This arrangement allowed simultaneous measurements of luminescence and fluorescence. Filling of the cuvette was completed in 200 msec; for an individual particle, the time lapse between mixing and appearence in view of the photomultipliers was about 40 msec. The bottom of the measuring compartment of the cuvettes consisted of a 2-mm layer of sintered glass situated 1 mm above the entrance to obtain uniform filling of the vessels. As mentioned earlier[12], the shortest filling time of the vessel that

could be obtained was 50 msec. We found, however, that this speed of mixing caused fluorescence artifacts which were absent when the longer filling time was used.

The photomultiplier for detecting the luminescence was situated close to the cuvette and equipped with a shutter (Ilex No. 4) and two glass filters, Schott RG 645, 3 mm and RG 630, 2 mm. The fluorescence, measured with the other photomultiplier was filtered by a Schott AL 688 interference and two RG 645 filters. The intensity ($3 \cdot 10^{-12}$ Einstein $\cdot$ cm$^{-2} \cdot$ sec$^{-1}$) of the fluorescence exciting light (472 nm) was too low to cause an increase in fluorescence yield even after 2 min of illumination. Delayed light seen by the second photomultiplier usually accounted for only a small part of the fluorescence signal, which was corrected for if necessary.

The photocurrents from both photomultipliers were amplified and recorded on a 2-channel Clevite Brush strip-chart recorder. Part of the delayed fluorescence signal was also fed into an electrical integrator, connected to a recorder which plotted the integrated light output.

All experiments were done at room temperature (20–22°).

## RESULTS

Some typical results obtained with the luminescence apparatus are shown in Fig. 2. The recordings in the left-hand column show similar characteristics to those

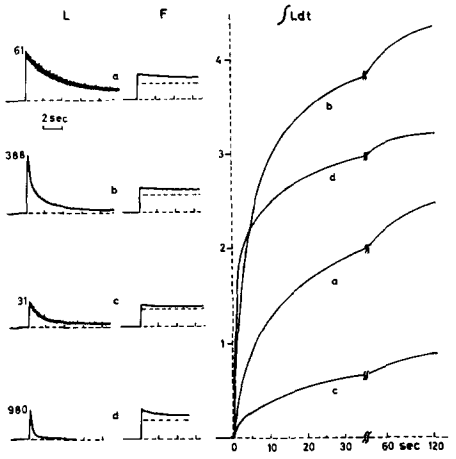


Fig. 2. Recordings of luminescence intensity ($L$), fluorescence yield ($F$) and integrated delayed light emission ($\int L dt$) of normal chloroplasts (see MATERIALS AND METHODS). The chloroplasts, suspended in 0.4 M sucrose and 0.05 M Tricine, brought to pH 7.8 with KOH, were preilluminated for 5 sec with saturating red light. The first mixing was with the same sucrose–Tricine buffer (Recordings a, b and c) or with a solution containing 0.4 M sucrose and 0.02 M succinic acid (d, pH after mixing 4.2). The second mixing, after a dark time of 3 sec, was with buffer (a), NaCl, final concentration 0.3 M (b), succinic acid–sucrose solution (c), or with a solution of 0.3 M sucrose and 0.1 M Tris (d, pH after mixing 8.7). The final chlorophyll concentration was $7.5 \cdot 10^{-5}$ M. Recording started immediately after the second mixing. The recordings of the left-hand row were made with different sensitivities of the apparatus; the numbers give the relative heights of delayed fluorescence immediately after the second mixing. The recordings for $F$ and $\int L dt$ are given on the same relative scales. The fluorescence recordings were corrected for a contribution caused by delayed fluorescence. This correction was 29% for the initial value of Recording d and correspondingly smaller for the other recordings. The dashed line indicates the fluorescence level after 2 min. The stimulation induced by mixing with NaCl varied with different batches of chloroplasts; it was usually 2–3-fold.

given in a previous paper[12]. Curve a shows the delayed fluorescence in a control experiment, where the chloroplasts were diluted twice with the same buffer; upon the second mixing, 3 sec after preillumination, the light emission was recorded (see MATERIALS AND METHODS). This signal represents what we shall call the "normal" delayed fluorescence of preilluminated chloroplasts. A six times higher signal (Curve b), was obtained when the second mixing was done with NaCl solution, and a 16-fold stimulation was observed (Curve d) when the suspension was first mixed with acid and subsequently with base. Mixing with acid alone resulted in a decreased luminescence signal (Curve c).

Addition of salt or acid–base treatment gave only an initial stimulation, as can be seen from the rapid decay, especially of the acid-base luminescence. This is also shown by the curves at the right-hand side of the figure, which give the total light emission, integrated over up to 2 min. The differences in total light output for Curves a, b and c are much smaller than for the corresponding initial luminescence levels at the left. The slopes of the curves show that the intensity of light emission for the acid–base experiment becomes lower than that of the control within 3 sec.

Recordings of the relative yield of chlorophyll fluorescence (middle column) showed much smaller differences than the luminescence recordings. Curve a shows the kinetics of fluorescence measured simultaneously with the luminescence for the control experiment. The fluorescence yield slowly decreased with time and after 2 min reached the level indicated by the dashed line. The latter level was approximately the same as that obtained without preillumination, and will be called "base" level. The difference between the total yield and the "base" yield will be called the "variable" fluorescence; at the beginning of the recording this amounted to about 55 % of the "base" fluorescence. The variable fluorescence is probably caused by $Q^-$, the primary electron acceptor of System 2 (ref. 16) which is reduced in the light and reoxidized in the dark. The base fluorescence and the initial yield of variable fluorescence were only slightly affected by salt or acid–base treatment. This indicates that the stimulated emission cannot be explained by changes in the concentration of $Q^-$ or in the fluorescence yield (see refs. 6–8), but is due to an increase in "intrinsic" delayed fluorescence, probably caused by a more rapid recombination of $Q^-$ and an oxidized product of the light reaction. The more rapid decay of the fluorescence yield shown by Recording d, suggesting a more rapid reoxidation of $Q^-$, would be in line with this assumption. For Experiment c there appears to be a correlation between the variable fluorescence, the luminescence signal and the total light emission which will be discussed later.

*Effects of suspension medium and of gramicidin*

Since preliminary experiments indicated that the intensity of the luminescence and stimulated luminescence were affected by the type of buffer in which the chloroplasts were suspended, we have studied these phenomena in Tris, Tricine and TES buffers.

Table I shows that the fluorescence yields of chloroplasts suspended in Tricine and Tris buffers (with or without added KCl) are basically the same. The fluorescence level in weak light (the base level, when Q is presumably largely oxidized), and in stronger light with 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU) (the maximal level, when Q is probably completely reduced) were approximately the same in both buffers. The main difference was in the fluorescence level in stronger light without

TABLE I

RELATIVE FLUORESCENCE YIELDS OF CHLOROPLASTS SUSPENDED IN DIFFERENT MEDIA

Class I chloroplasts were isolated in Tricine–KOH buffer, (see MATERIALS AND METHODS) and suspended in the various buffers indicated (pH 7.8). The concentration of $K^+$, if present, was 15 mM, that of chlorophyll was $5 \cdot 10^{-5}$ M. Fluorescence was excited by weak modulated blue light (472 nm, $4 \cdot 10^{-11}$ Einstein$\cdot$cm$^{-2}\cdot$sec$^{-1}$). The yield of fluorescence was modified by additional actinic light (487 nm, $1 \cdot 10^{-9}$ Einstein$\cdot$cm$^{-2}\cdot$sec$^{-1}$) in a way similar to that described by DUYSENS AND SWEERS[16]. The steady-state fluorescence yield without actinic light is denoted by $f_0$; $f_1$ denotes the steady-state yield with actinic light, and $f_{(2\,sec)}$ the yield, 2 sec after cessation of actinic illumination. The fluorescence yields are expressed in arbitrary units, different from those of the other tables and figures.

| *Medium* | *No additions* | | | $1 \cdot 10^{-6}$ *M DCMU* | | |
|---|---|---|---|---|---|---|
| | $f_0$ | $f_1$ | $f_{(2\,sec)}$ | $f_0$ | $f_1$ | $f_{(2\,sec)}$ |
| Tricine–KOH | 9.4 | 18.9 | 14.1 | 10.0 | 28.7 | 17.3 |
| Tris–HCl + KCl | 9.7 | 29.7 | 17.9 | 11.7 | 30.5 | 22.6 |
| Tris–HCl | 9.7 | 24.4 | 17.5 | 12.1 | 30.2 | 22.9 |

DCMU, which indicated a higher level of reduction of Q in Tris than in Tricine buffer. Upon cessation of illumination the fluorescence level decreased to about half the level in weak light in about 2 sec in most tests. The observation that the base and the maximal fluorescence yield were almost independent of the type of buffer used indicates that the yields of variable fluorescence in the following tables can be compared in terms of oxidation–reduction levels of Q.

Table II shows the effect of suspension medium on luminescence. The main difference between chloroplasts suspended in TES and Tris buffers was in the stimulation induced by NaCl, which was larger in TES buffer. The controls, and considering the smaller pH jump in the case of the TES experiment, the acid–base signals were of

TABLE II

LUMINESCENCE AND FLUORESCENCE WITH DIFFERENT BUFFERS

Normal chloroplasts were isolated in TES–KOH buffer (pH 7.8) (see MATERIALS AND METHODS), and suspended either in 0.05 M TES–KOH (pH 7.8) or in 0.05 M Tris–HCl (pH 7.8) with added KCl ($K^+$ concn., 30 mM). In the acid–base experiment the pH was lowered to 5.6 (TES experiment) and 4.2 (Tris experiment) respectively, by mixing with an equal volume of a sucrose solution containing 0.02 M succinic acid; during the second mixing the pH was subsequently raised to 8.5 and 8.6 respectively with 0.1 M Tris (chlorophyll concentration, $2.5 \cdot 10^{-5}$ M). The 3rd column gives the initial intensity of luminescence after mixing (*cf.* Fig. 2), the 4th column the base fluorescence, measured after 2 min darkness, the 5th column the variable fluorescence after mixing, and the 6th column the total luminescence emission, integrated over 2 min. The numbers in parentheses refer to experiments with $1 \cdot 10^{-6}$ M gramicidin D, added 10 min before the measurement. See Fig. 2 for the other conditions. The luminescence is expressed in the same units as in Fig. 2.

| *Type of experiment* | *Buffer* | *Luminescence* | $F_0$ | $\Delta F$ | *Integrated luminescence* |
|---|---|---|---|---|---|
| Control | TES–KOH | 65 (59) | 16.0 (17.0) | 3.0 (4.0) | 119 (110) |
| NaCl addition | TES–KOH | 155 (130) | 18.0 (18.5) | 5.0 (6.5) | 172 (142) |
| Acid–base | TES–KOH | 500 (610) | 18.0 (18.0) | 5.0 (5.5) | 143 (97) |
| Control | Tris–HCl + KCl | 70 (69) | 20.0 (21.0) | 7.5 (8.0) | 120 (116) |
| NaCl addition | Tris–HCl + KCl | 88 (151) | 22.0 (23.0) | 7.0 (8.0) | 173 (173) |
| Acid–base | Tris–HCl + KCl | 980 (860) | 22.0 (21.0) | 7.5 (6.0) | 141 (108) |

comparable magnitude. Gramicidin had little effect upon the base and variable fluorescence. With Tris the NaCl- and KCl-induced luminescence was stimulated by gramicidin, as was reported earlier[12], but in TES buffer a slight inhibition occurred, both in the salt-induced and control luminescence signals. In Tris buffer without KCl, even larger stimulations of the salt-induced signals were induced by gramidicin. The acid–base signal was slightly stimulated in TES and inhibited in Tris buffer.

Results obtained with chloroplasts suspended in Tricine–KOH buffer were similar to those with TES-KOH. With each buffer tested, the potassium benzoate signal was about two fold stimulated by gramicidin. The stimulation by valinomycin of the luminescence signal induced by KCl and potassium benzoate, reported earlier[12], was observed with all buffers.

The differences between the results with Tris and the other buffers are difficult to explain, but are probably related to the well-known uncoupling action of Tris. The slightly inhibitory action of gramicidin, which is thought to enhance cation permeabilities, on both the normal and NaCl-induced luminescence with TES may be explained by a lowering of an electrochemical potential over the thylakoid membrane, resulting from the preillumination. With Tris no such inhibition occurred, suggesting that the electrochemical potential had already disappeared as a result of the action of Tris alone. The different effect of gramicidin on the normal luminescence was much more conspicuous after a shorter dark time (0.1 sec) as shown in Table III. Here, the luminescence signal was considerably higher in Tricine than in Tris buffer. Gramicidin gave a more than 2-fold inhibition of the signal in Tricine, but had a much smaller effect on the luminescence in Tris buffer. The results in Table III give a clear example of the absence of a simple correlation between the yields of delayed and prompt fluorescence as can be seen *e.g.* by comparison of lines 1 and 2 or some of the columns.

The striking difference between the effects of gramicidin on the NaCl-induced signals in TES and Tris buffer is not easily explained. The stimulation could be due to an enhanced permeability of $Na^+$, resulting in a higher membrane potential upon

TABLE III

EFFECTS OF GRAMICIDIN ON LUMINESCENCE AND FLUORESCENCE

Chloroplasts were prepared as mentioned for Table I. The chloroplast suspension was preilluminated at an intensity of $6 \cdot 10^{-10}$ Einstein$\cdot$cm$^{-2}\cdot$sec$^{-1}$ in a transparent vessel, situated at the place of the measuring cuvette. After a dark time of 0.1 sec, a shutter in front of the photomultiplier was opened, and the luminescence intensity was measured. The fluorescence yield was measured in a separate experiment with the same photomultiplier. The fluorescence is expressed in units different from those in Table II. $\Delta F$ denotes the difference in fluorescence yields measured after 0.1 sec and 2 min. Concentration of chlorophyll, $5 \cdot 10^{-5}$ $M$; of gramicidin D, $1 \cdot 10^{-6}$ M. Further conditions as for Table II. The other symbols have the same meaning as for Table II, except that integrated luminescence was recorded over 100 sec.

| *Medium* | *Luminescence* | | *Integrated lum.* | | $F_0$ | | $\Delta F$ | |
|---|---|---|---|---|---|---|---|---|
| | *No gramicidin* | *With gramicidin* | *No gramicidin* | *With gramicidin* | *No gramicidin* | *With gramicidin* | *No gramicidin* | *With gramicidin* |
| Tricine–KOH | 4120 | 1650 | 1270 | 810 | 8.0 | 7.7 | 6.7 | 7.0 |
| Tris–HCl + KCl | 1570 | 1120 | 960 | 760 | 9.0 | 8.5 | 14.5 | 14.5 |
| Tris–HCl | 2180 | 2490 | 930 | 930 | 9.0 | 8.0 | 11.3 | 11.0 |

salt addition, but this explanation does not account for the absence of a stimulation in TES buffer.

*pH dependence*

As was shown in Fig. 2, a decrease of pH after preillumination caused a decrease of delayed fluorescence. Fig. 3a shows the results of a series of experiments in which the chloroplasts, prepared in Tricine–KOH, were subjected to sudden pH jumps by mixing with various concentrations of 2-(*N*-morpholino)ethanesulfonic acid (MES)–HCl or Tricine–KOH buffers. An increase of pH, up to pH 11, caused an increase in delayed fluorescence, and a decrease, down to pH 3, caused an inhibition. Decreasing to pH below 3 caused a strong temporary stimulation of delayed fluorescence. The same results were obtained when the MES–HCl was partly replaced by succinic acid. The variable fluorescence was affected qualitatively in a similar way, but less strongly than the delayed fluorescence. The level of base fluorescence was almost independent of pH.

It thus appears that part of the effect of pH on delayed fluorescence is due to a change in efficiency of fluorescence quenching by $Q^-$, perhaps caused by a pH-dependent equilibrium between two reduced forms of Q. The effect may also be partly caused by the pH gradient generated, which is favorable for light emission when the pH outside of the chloroplast lamellae is increased, but unfavorable when it is lowered. The stimulation at very low pH may be due to damage of the lamellar structure.

Fig. 3b shows experiments with Tris buffer. The results were similar to those with Tricine, except that stimulation occurred even below pH 4.5, the stimulation in the region above pH 7.8 was smaller, and was absent in the integrated luminescence signal, perhaps because of an uncoupling action by Tris. MILES AND JAGENDORF[11], also using Tris buffer, reported an enhancement of luminescence only upon lowering

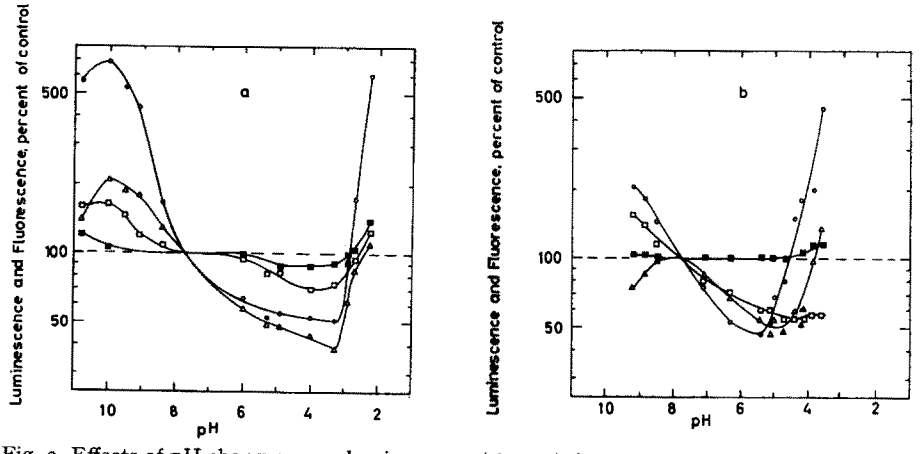


Fig. 3. Effects of pH changes upon luminescence (○—○), integrated luminescence (△—△), base fluorescence (■—■) and variable fluorescence (□—□). The base fluorescence gives the fluorescence yields without preillumination. Initial luminescence and fluorescence were measured immediately after mixing with the solutions indicated below, as described for Fig. 2. (a) Class I chloroplasts in 0.01 M Tricine–KOH buffer (pH 7.8). The pH was lowered with MES–HCl, or increased with Tricine–KOH. Dark time was 2 sec. Final chlorophyll concentration $2.5 \cdot 10^{-5}$ M. (b) Normal chloroplasts in 0.05 M Tris–HCl buffer (pH 7.8). The pH was lowered with succinic acid, or increased with Tris. Dark time was 3 sec. Chlorophyll concentration, $9 \cdot 10^{-5}$ M. The osmolarity of the suspensions was kept constant at 0.4 M with sucrose throughout the experiments.

the pH, possibly because of different measuring techniques and experimental conditions. Gramicidin D stimulated the delayed fluorescence in acid medium. At $1 \cdot 10^{-6}$ M it gave a stimulation by 50 % at pH 5.6 in TES medium and of 70 % at pH 4.2 in Tris medium.

*Concentration and permeability effects*

Provided certain conditions are met (*e.g.* passive and independent ion transport, the absence of a nett electric current) and when only monovalent ions are present, the membrane potential is given by the so-called Goldman equation[17]:

$$E_{(i-o)} = \frac{RT}{F} \ln \frac{p_{C_1}[C_1^+]_o + p_{C_2}[C_2^+]_o + \ldots p_{A_1}[A_1^-]_i + \ldots}{p_{C_1}[C_1^+]_i + p_{C_2}[C_2^+]_i + \ldots p_{A_1}[A_1^-]_o + \ldots} \quad (1)$$

where $p_C$ and $p_A$ are the permeability coefficients for the cations ($C^+$) and anions ($A^-$), respectively, the terms between brackets denote outside and inside ion concentrations (activities), and the other symbols have their usual meanings.

The membrane potential developed over the thylakoid membrane immediately after addition of a monovalent salt $C^+A^-$ may be written as:

$$E = \frac{RT}{F} \ln \frac{a + p_C[C^+]_o}{b + p_A[A^-]_o} \quad (2)$$

where $a$ and $b$ are determined according to Eqn. 1 by the products of the permeability coefficients and the concentrations of ions inside and outside the membrane before salt addition, and $[C^+]_o$ and $[A^-]_o$ are the concentrations of the added ions outside of the thylakoid.

As the equation shows, the membrane potential increases upon addition of salt if $p_C > p_A$; the quotient after the logarithm increases linearly with the concentration when $b >> p_A\,[A^-]_o$, as occurs with low concentrations of added salt or when $p_A$ is small. With potassium benzoate, which was assumed to have a low $p_A$, such a linear relation was observed[12] between the intensity of the stimulated luminescence and the concentration, up to a high concentration. This suggested that the intensity of emission is proportional to the exponential of the membrane potential. With KCl, in contradistinction to observations of MILES AND JAGENDORF[11], we observed a saturation of the emission intensity below 0.1 M. This could be explained by the assumption of a $p_A$ for $Cl^-$, which although smaller than of $K^+$, is nevertheless of the same order of magnitude. At relatively high concentrations of salt, $a$ and $b$ then become small compared with the other terms.

When a mixture of two salts, $C^+A^-$ and $C^+B^-$, is used slight modification of Eqn. 2 predicts that, if $p_A$ is small compared with the other permeability coefficients, and the concentration of $C^+A^-$ is not too low, the membrane potential developed upon adding a solution containg both salts will be lower than that obtained upon adding a solution of $C^+A^-$ at the same concentration alone, because of a large term $p_B[B^-]_o$ in the denominator.

Experiments with mixtures of KCl and potassium benzoate showed that the luminescence stimulation by potassium benzoate was decreased by KCl. Qualitatively this is in agreement with the assumption[12] that the permeability for $Cl^-$ is much larger than for benzoate ions. However, the decrease of stimulation was smaller than

would be expected. *E.g.* mixing with 150 mM potassium benzoate and 150 mM KCl gave a signal which was only 20% lower than that upon mixing with 150 mM potassium benzoate alone, and still 4.8 times that upon mixing with 150 mM KCl alone. Even if one assumes that the permeability of the benzoate ion is negligible compared with that of $Cl^-$, then the quotient after the logarithm of Eqn. 2, and thus perhaps the signal with benzoate and $Cl^-$ should have been only twice that with KCl. This suggests that the stimulation by benzoate is partly due to effects other than the establishment of a membrane potential (see DISCUSSION).

We also investigated the effects of KCl and valinomycin upon the NaCl and acid–base induced stimulation of delayed fluorescence (Table IV). Preincubation with KCl in the presence of valinomycin can be expected to lower the absolute size of the membrane potential by exchange of $K^+$ for other cations ($Na^+$ or $H^+$) upon addition of salt or base. This can also be seen from the Goldman equation: in the presence of the highly permeable $K^+$–valinomicin complex, the change in membrane potential upon mixing with salt or base will be reduced because of higher terms $a$ and $b$ in Eqn. 2.

TABLE IV

EFFECTS OF KCl AND VALINOMYCIN UPON STIMULATED DELAYED FLUORESCENCE

The experiments were done with normal chloroplasts, prepared and suspended in TES–NaOH buffer (pH 7.8). Valinomycin and KCl were added at concentrations of $2 \cdot 10^{-6}$ M and 10 mM, respectively, before the preillumination. The final chlorophyll concentration was $7 \cdot 10^{-5}$ M. The other conditions and the units used were as for Table II.

| *Type of Experiment* | *No addition* | *Valinomycin* | *KCl* | *KCl + valinomycin* |
|---|---|---|---|---|
| Control | 73 | 70 | 82 | 74 |
| Acid–base | 260 | 280 | 310 | 465 |
| NaCl | 150 | 107 | 195 | 71 |
| Sodium benzoate | — | — | 300 | 170 |

This predicted effect is in agreement with the lowering of the NaCl signal by the presence of KCl and valinomycin. The lowering by valinomycin alone can be explained by the presence of a small concentration of endogenous $K^+$. The luminescence induced by sodium benzoate was also depressed by valinomycin in the presence of KCl. The acid–base experiments showed a considerable stimulation by KCl and valinomycin, and a small one by either component alone. If it is assumed that this effect is also due to the lowering in absolute size of a membrane potential, generated upon mixing with base, then these observations indicate that this potential is positive on the outside of the membrane. Presumably generated by efflux of protons, the potential would, because of its sign, tend to depress the stimulation of light emission brought about by the pH difference. Lowering the membrane potential by KCl and valinomycin then relieves this inhibition.

The "normal" delayed light, as well as the base and variable fluorescence (not shown in Table IV), were not affected by KCl and valinomycin.

*Detergent treatment*

Fig. 4 shows the effects of incubation of chloroplasts with increasing concentrations of Triton X-100. The various types of luminescence phenomenon were affected differently by the detergent. In contrast to the "normal" delayed fluorescence, which was relatively resistant towards detergent treatment, the acid–base induced luminescence was strongly decreased by low concentrations of Triton. Most of the signal due to salt addition was about equally sensitive, but some salt-induced stimulation of luminescence persisted even at much higher concentrations.

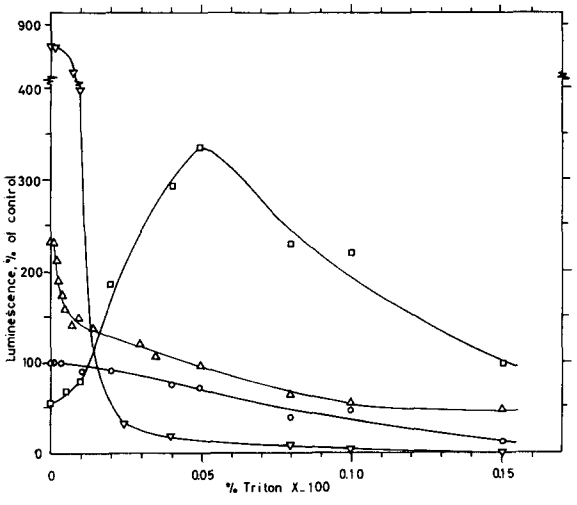


Fig. 4. Effect of different concentrations of Triton X-100 upon various types of luminescence. Class I chloroplasts (chlorophyll concentration, $1 \cdot 10^{-4}$ M) were suspended in a solution of 0.4 M sucrose and 0.01 M Tricine–KOH (pH 7.8), and preincubated for 5 min at room temperature with the concentration of Triton indicated. A concentration of 0.10% correponds to a molar ratio of Triton to chlorophyll of 15.9. ○—○, normal delayed light; △—△, NaCl-induced luminescence; □—□, luminescence upon acidification with MES–HCl to a final pH of 4.6; ▽—▽, acid–base-induced luminescence (final pH 8.8 obtained by mixing with Tricine–KOH. The dark time after preillumination was 2 sec. Further conditions as for Fig. 2.

Measurement of the activity of the Hill reaction with ferricyanide for the same chloroplast preparations (Fig. 5) at various concentrations of Triton gave similar results to those reported by VERNON AND SHAW[18]. The stimulation of electron transport, maximal at 0.02 % Triton, has been attributed to uncoupling of photophosphorylation[18], the inhibition at higher concentrations to destruction of the oxygen-evolving system. The uncoupling of phosphorylation, the inhibition of acid–base luminescence and the partial inhibition of the NaCl effect are probably due to disruption of the thylakoid lamellae. Parallel measurements showed that the light-induced increase in absorption at 515 nm, thought to be an indicator of membrane functioning[19], was completely inhibited at a Triton concentration of 0.01%. In contrast to the slow phase of "normal" delayed fluorescence, which was apparently largely independent of the integrity of the thylakoid, the rapid phase, measured 5–10 msec after preillumination by means of a phosphorescope, was very sensitive to detergent treatment (Fig. 5).

Disruption of the thylakoid membrane gave a strong stimulation of delayed fluorescence upon acidification, probably due to easier access of acid to the inner side

of the membrane. The effect may be related to the strong inhibition of the acid–base signal. Apparently most of the energy stored during preillumination had been dissipated upon acidification alone.

In agreement with observations of SAUER AND PARK[20] Triton, at concentrations above about 0.02 %, was found to enhance considerably the fluorescence yield. This complicates a comparison of the yield of delayed fluorescence at high concentrations.

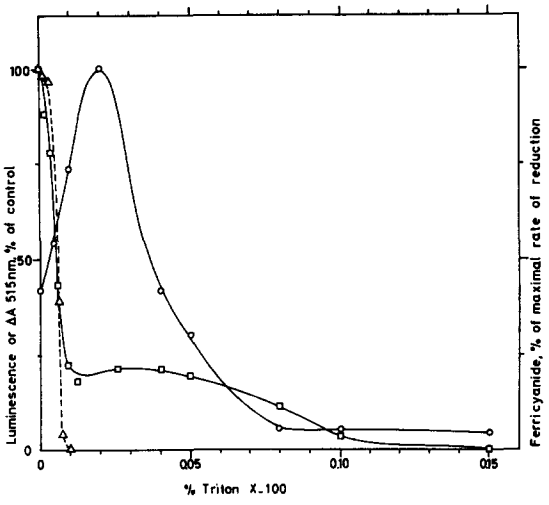


Fig. 5. Effect of Triton X-100 upon the rapid phase of delayed fluorescence (□—□), ferricyanide Hill reaction (○—○) and absorption increase at 515 nm (△—△). Delayed fluorescence was measured with a Becquerel phosphoroscope. The wavelength of exciting light was 481 nm, the intensity $3 \cdot 10^{-9}$ Einstein·cm·$^{2}$ sec$^{-1}$, the average dark time 5 msec. Ferricyanide reduction was measured spectrophotometrically as the initial rate of reduction in saturating light of 656 nm ($12 \cdot 10^{-9}$ Einstein·cm·$^{2}$ sec$^{-1}$); without Triton the reduction rate was 40 μmoles/mg of chlorophyll per h. The concentration of ferricyanide was $5 \cdot 10^{-4}$ M. The steady-state absorption increase at 515 nm was measured with the same intensity and wavelength of actinic light. The other conditions were as for Fig. 4.

*Heating*

Short heating at 45° affected the various types of delayed fluorescence signals differently. With normal chloroplasts, suspended in Tris–sucrose buffer, after 1 min of heating the normal luminescence was decreased to 24 % of its original value. KCl-induced stimulation had disappeared, whereas the acid–base signal was reduced somewhat less (by 70 %) than the normal luminescence. The delayed light signal upon acidification (to pH 4.6) was less sensitive, resulting in a more than two-fold stimulation with respect to the control experiment after 1 min of heating.

## DISCUSSION

The experiments described in this and in the previous paper[12] strongly indicate that the phenomenon of stimulated luminescence is closely related to the functioning of the thylakoid membrane. The evidence reported, including the effects of agents such as valinomycin, which change the membrane permeability of ions, and the experiments with mixtures of salts and with the detergent Triton X-100, indicate that

the stimulation of delayed fluorescence is brought about by the generation of an electrochemical potential across the thylakoid membrane.

There are several indications (*e.g.* refs. 1, 2) that most or all of the delayed fluorescence is emitted by the chlorophyll *a* of Photosystem 2, and it has been assumed[7] that the light emission originates from a back reaction of the reduced and oxidized reactants of Photosystem 2. The simultaneous measurements of delayed and prompt fluorescence reported in this paper indicate that the stimulation of delayed fluorescence by acid–base or salt treatment is not paralleled by a significant increase in prompt fluorescence. Therefore, we conclude that the luminescence stimulation reflects primarily an increase of the rate of the back reaction between the primary photoproducts, which is not due to an increase in the concentration of the reduced electron acceptor $Q^-$, caused *e.g.* by "reversed" electron flow from other intermediates in the photosynthetic chain between System 1 and 2. In agreement with this conclusion we found that DCMU ($5 \cdot 10^{-5}$ M), which is thought to inhibit electron flow between Q and these other components[16], gave only about 50 % inhibition of stimulated luminescence, in accordance with observations of MILES AND JAGENDORF[11]. The fact that ATP formation upon salt addition has not been observed[11] and is moreover energetically improbable[12] is also in line with the above reasoning.

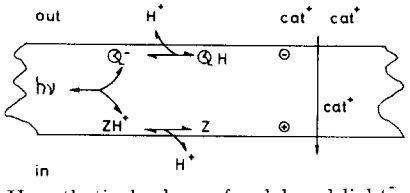


Fig. 6. Hypothetical scheme for delayed light emission as discussed in the text.

In view of the above considerations which suggest that the salt- or acid–base-induced stimulation of light emission is due to a direct effect upon the reaction rate of the primary photoreactants, we propose the following scheme to account for the observations reported (see Fig. 6). Denoting the primary electron donor by ZH, we may write the photoreaction of System 2 as:

$$ZH + Q \xrightarrow{h\nu} ZH^+ + Q^- \qquad (1)$$

ZH and Q are thought to be located on the inside and outside of the thylakoid membrane, as was also assumed by WITT *et al.*[19] on the basis of other evidence. We further assume that $ZH^+$ and $Q^-$ react in a pH-dependent equilibrium, formally written as

$$ZH^+ \rightleftharpoons Z + H^+ \qquad (2)$$

and

$$Q^- + H^+ \rightleftharpoons QH \qquad (3)$$

Delayed fluorescence occurs upon reversal of Reaction 1. Recent evidence indicates that Z may exist in four oxidation states, $Z^+$, $Z^{2+}$, $Z^{3+}$ and $Z^{4+}$, of which $Z^{3+}$ is the main reactant in producing luminescence[21], but this does not affect the basic formulation of the scheme, since ZH in Reaction 1 would then represent the twice-oxidized form.

The acid–base-induced stimulation of luminescence occurs upon the establishment of a temporary pH gradient such that the pH inside the thylakoid is considerably lower than outside. This would favor a shift of the equilibrium of Reactions (2) and (3) towards the formation of $ZH^+$ and $Q^-$, and consequently enhance the rate of reversal of Reaction (1) and stimulate emission of light. Acidification alone would tend to produce the opposite effect, because Reaction (3) would be shifted to the right. The effect is apparently not fully compensated by the production of $ZH^+$ which may be assumed to occur when the acid subsequently penetrates into the thylakoid. The lowering of the variable fluorescence yield upon acidification suggests that QH is a somewhat more effective quencher of chlorophyll fluorescence than $Q^-$.

As was discussed above and elsewhere[12], addition of salts such as NaCl and KCl probably produces a diffusion potential which is positive on the inside with respect to the outside of the thylakoid membrane. It is conceivable that a potential of this sign accelerates the reaction between the two charged species $ZH^+$ and $Q^-$ located on opposite sides of the membrane. The experiments with KCl and valinomycin reported in the previous section suggest that acid–base treatment generates a negative potential which counteracts the stimulation caused by the pH gradient. Valinomycin then relieves this inhibition by reducing this potential.

The mechanism discussed above also presents an explanation for the observed rapid decay[2,22] in intensity of delayed light shortly after preillumination, which is known to decay about a 100-fold between $1 \cdot 10^{-3}$ and $1 \cdot 10^{-1}$ sec. This fast decay is remarkable in view of the much slower disappearance of reduced Q as indicated by the decrease of the fluorescence yield in the dark (*e.g.* Tables I and II), and the various oxidized forms of Z, as deduced by JOLIOT *et al.*[23] from measurements of oxygen evolution. However, according to the theories of MITCHELL[24] and WITT *et al.*[19], the preillumination would also generate an electrochemical potential difference, consisting of a positive membrane potential and a pH difference. Both these would, according to the scheme presented here, strongly stimulate light emission. The stimulation factor then decreases upon decay of the membrane potential and proton gradient. The membrane potential generated in the light disappears almost completely within 1 sec, as judged from the decay of the absorption change at 515 nm (ref. 19) which is thought to be its indicator.

The above explanation also accounts for the much higher sensitivity towards Triton treatment of the rapid than of the slow phase of delayed fluorescence. It also explains why gramicidin and other uncouplers inhibit the rapid phase (see ref. 25 and Table III) of delayed light much more strongly than the slow phase. Gramicidin also enhances the decay of the 515 nm change[19].

The insensitivity to gramicidin of stimulated luminescence is in contrast to observations of MAYNE[10]. However, the apparent discrepancy is probably due to the much longer acidification period applied, during which time much of the energy stored may have dissipated, owing to the luminescence stimulation by gramicidin at low pH (see section *pH dependence*). This also applies to similar observations by MILES AND JAGENDORF[11]. The stimulation that was also observed with other uncouplers, 2,4-dinitrophenol and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, is probably due to an enhanced permeability of $H^+$.

With gramicidin, the salt- and acid–base-induced signals decayed more rapidly than without this uncoupler, as can be seen from the integrated signals of Table II.

A typical acid–base experiment in TES buffer gave a 2-fold increase in the rate of decay by gramicidin. This suggests that the pH difference disappears more rapidly.

The inhibition of luminescence by heating is probably due to inactivation of a photochemical reaction[7]. However, the different sensitivity of the various types of delayed fluorescence suggests that the properties of the thylakoid membrane are also affected. The effect may be related to results obtained by EMMETT AND WALKER[26], which suggested changes in membrane permeabilities (uncoupling) induced by heating.

The relatively strong stimulation by benzoate, which is much larger than with other anions[12] and appears to be somewhat unique, has been explained by a very low permeability of the benzoate anion[12], in contrast to what has been concluded for related anions in the case of synthetic lipid membranes[27]. Evidence that this hypothesis is only partly right has been given in this paper. Another explanation might be that part of the effect is caused by penetration of the free acid into the thylakoid, causing a decrease of pH favorable for light emission*.

The scheme discussed above is hypothetical and probably not the only one that may explain the observed phenomena. However, it may be a useful working hypothesis for further experiments.

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